Original Article

IN VITRO ANTIPLASMODIAL AND CYTOTOXIC ACTIVITIES OF A SUNGKAI (*PERONEMA CANESCENS*) LEAF EXTRACT

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ABSTRACT

Objective: The aim of this study was to assess the antiplasmodial and cytotoxic activities and to evaluate the selectivity indices of acetone, ethanol and aqueous extracts of *Peronema canescens* leaves.

Methods: Antiplasmodial activity was measured *in vitro* against *Plasmodium falciparum* strains D10 and FCR3 by 72 h incubation at 37 °C in a candle jar. Parasitaemia was calculated by counting the parasite numbers in thin smears. *In vitro* cytotoxicity was assayed in Vero cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and reading the absorbency at 595 nm with an ELISA reader. The assessed parameters included: 50% inhibitory concentration (IC_{50}) of antiplasmodial activity, IC_{50} of cytotoxic activity and the selectivity index of the *Peronema canescens* leaf extract.

Results: The IC_{50} values for the acetone, ethanol and aqueous extracts were 26.33±1.65, 37.96±8.17 and 12.26±1.05 µg/ml, respectively, against the *Plasmodium falciparum* D10 strain and 51.14±8.65, 70.22±14.13 and 34.85±6.04 µg/ml, respectively, against the FCR3 strain. For Vero cells, the IC_{50} values for the acetone, ethanol and aqueous extracts were 23.37±5.63, 629.46±24.85 and 634.00±144.82 µg/ml, respectively. The selectivity indices of these extracts were 0.89, 16.46 and 51.70, respectively, for the D10 strain and 0.46, 8.90 and 18.00, respectively, for the FCR3 strain.

Conclusion: The aqueous extract of *Peronema canescens* leaves had the highest *in vitro* antiplasmodial activity and the best selectivity index.

Keywords: *Peronema canescens*, Antiplasmodial activity, MTT method, Selectivity index, Cytotoxicity index, *Plasmodium falciparum*

INTRODUCTION

Malaria is an endemic disease in Indonesia: in 2016, the annual parasite incidence (API) was 0,77 per 1000 population. *Plasmodium falciparum*, one of the pathogens responsible for this disease. The drug of choice for malaria treatment is artemisinin-based combination therapy (ACT). However, the latest studies have shown a gene mutation in *Plasmodium falciparum* that is associated with a reduced response to ACT [1–5].

This new resistance to the malarial plasmodium has initiated the development of new antimalarial drugs. Plant-based medicines are viewed as one of the alternative sources of new antimalarials. In Indonesia, a number of different plants have been studied for their antimalarial properties, including papaya leaves (*Carica papaya* Linn), mahogany fruit (*Swietenia mahagoni* Jacq.), neem leaves (*Azadirachta indica* Juss.), pule seeds (*Alstonia scolaris*), meniran herb (*Phyllanthus niruri* L.), Mundus bark (*Garcinia dulcis* Roxb.), *Carthamus tinctorius* Linn. flowers, *Artocarpus* sp. and sambiloto (*Andrographis paniculata*) [6–12]. One plant that has been used empirically as an antimalarial in some communities in southern Sumatera is sungkai (*Peronema canescens*), although research on its antiplasmodial activity is still limited. One previous study reported that a sungkai leaf extract showed *in vivo* antiplasmodial activity in Swiss mice [13]. Other studies published in 2004 and 2005 showed that sungkai leaf extracts had *in vitro* antibabesia activity [8, 14].

Natural ingredients, like those in sungkai leaf extracts, should go through thorough testing, prior to their use as drugs, to assess their activity as well as their toxicity. Thus far, sungkai leaf extracts have undergone only limited testing; therefore, the aim of the present study was to assess the activity and toxicity of various extracts of sungkai leaves prepared using solvents with different polarities. We investigated the antiplasmodial and toxicity activities and evaluated the selectivity or cytotoxicity indices of acetone, ethanol and aqueous extracts of *Peronema canescens* leaves.

MATERIALS AND METHODS

Chemicals and reagents

Acetone, ethanol and distilled water, RPMI 1640 (Sigma-Aldrich, St. Louis MO, USA) supplemented with 25 mmol HEPES (Sigma) and 30 mmol $NaHCO_3$, gentamicin, human erythrocytes (O blood type), human serum (O blood type), sorbitol, Giemsa solution, M199 (Sigma) medium with 10 % foetal bovine serum and 0.5–1% fungizon, thiazolyl blue tetrazolium bromide (MTT = 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma), sodium dodecyl sulphate (SDS) (Sigma) in 0.01 M HCl.

Plant materials and extracts

Leaves were collected from *Peronema canescens* plants growing in their natural habitat in the Lahat and Empat Lawang Districts, South Sumatra Province, Indonesia. The location coordinates where the specimens of *Peronema canescens* leaves were growing were 3 °42'6.15"S–103 °19'10.43"E and 3° 37'23.14"S–103 °3'36.31"E. The plant was identified at the Pharmacognocy Laboratory, Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta with number UGM/FA/166/Ident/XII/2006.

The *Peronema canescens* leaves were air dried and ground to a fine powder. A percolation method was used to obtain the *Peronema canescens* leaf extracts from the leaf powder using acetone, ethanol and distilled water solvent. The powder was first percolated with acetone and obtain the acetone extract. The residue from the acetone extract was then percolated with absolute ethanol to obtain the ethanol extract. The residue from the ethanol extract was then percolated with distilled water to obtain the aqueous extract. The

filtrates were concentrated with a rotary evaporator to generate the final acetone, ethanol and aqueous extracts [15, 16].

In vitro cultivation of *Plasmodium falciparum*

Plasmodium falciparum strains D10 and FCR3 were obtained from continuous cultures at the Parasitology Laboratory, Department of Parasitology, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University. Both strains were cultured according to Trager and Jensen [17, 18] and were maintained in vitro in human erythrocytes (O blood type) with a 1% haematocrit in RPMI 1640 (Sigma) supplemented with 25 mmol HEPES (Sigma) and 30 mmol NaHCO$_3$. Gentamicin at 25 mg/l and 10% human serum (O blood type) were also added, and the parasite cultures were incubated at 37 °C in candle jars. The media were changed every day. The parasite cultures were synchronised with 5% sorbitol, as described by Lambros and Vanderberg [9, 16, 18].

In vitro antiplasmodial activity

In vitro antiplasmodial activity was tested in the parasitology laboratory, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University using 96-well microplates. The ring stage of the *Plasmodium falciparum* cultures with 2% parasitaemia and 3% haematocrit was used in this test, and the tests were performed three times for each sample (triplicate). A total of 100 µl of parasite culture and 100 µl of leaf extract in the culture medium were distributed into each well of the microplate. The leaf extracts were supplied at varying concentrations. The parasite and extract mixtures were incubated for 72 h at 37 °C in the candle jar. Parasitaemia was calculated after incubation by counting the parasite numbers in thin smears. The parasite growth inhibition was expressed as a percentage of the negative control. The antiplasmodial activity was determined by calculating the IC$_{50}$ [9, 16].

Cytotoxicity activity

In vitro cytotoxicity activity tests were also conducted at the parasitology laboratory, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University. The Vero cells used in this study were obtained from the continuous culture at Parasitology

Laboratory, Department of Parasitology, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University. The cells were cultured in M199 (Sigma) medium with 10% foetal bovine serum and 0.5–1% fungizon at 37 °C and 5% CO$_2$. The Kasugai method, with some modification, was used to measure cytotoxicity [19]. A 1 µl volume containing of 1×10^4 Vero cells in culture medium was distributed into a 96-well plate and incubated for 24 h at 37 °C, 5% CO$_2$. A 100 µl volume of leaf extract at various concentrations in culture medium was then added and incubated for a further 72 h at 37 °C, 5% CO$_2$. The culture medium was then collected with a micropipette, and 100 µl was mixed with 10 µl MTT solution (Sigma) and incubated for 4 h at 37 °C, 5% CO$_2$. A 100 µl volume of 10% SDS in 0.01 M HCl was added to dilute the formazan and incubate for 18 h at room temperature. The absorbance was read with an ELISA reader at 595 nm. The IC$_{50}$ values was calculating with probity analysis [9, 16, 19].

Statistical analysis

The antiplasmodial and cytotoxicity activities of the three extracts tested were compared using one-way ANOVA. The IC$_{50}$ values were determined using probit analysis.

RESULTS

In vitro antiplasmodial activity

The highest concentrations of the acetone, ethanol and aqueous extracts inhibited the growth of *Plasmodium falciparum* strain D10 by 85.25±2.43%; 82.38±4.24% and 87.32±2.77% after a 72 h incubation, as shown in fig. 1. The mean IC$_{50}$ values for the acetone, ethanol and aqueous extracts were 26.33±1.65, 37.96±8.17 and 12.26±1.05 µg/ml, respectively. The ANOVA test (p = 0.05) for the D10 strain revealed a significant difference between the three extracts (p = 0.00). *Post-hoc* test results showed significant differences between the acetone and aqueous extracts (p = 0.03) and between the ethanol and aqueous extracts (p = 0.01), but no significant differences between the acetone and ethanol extracts (p = 0.06) in terms of their ability to inhibit the growth of the D10 strain.
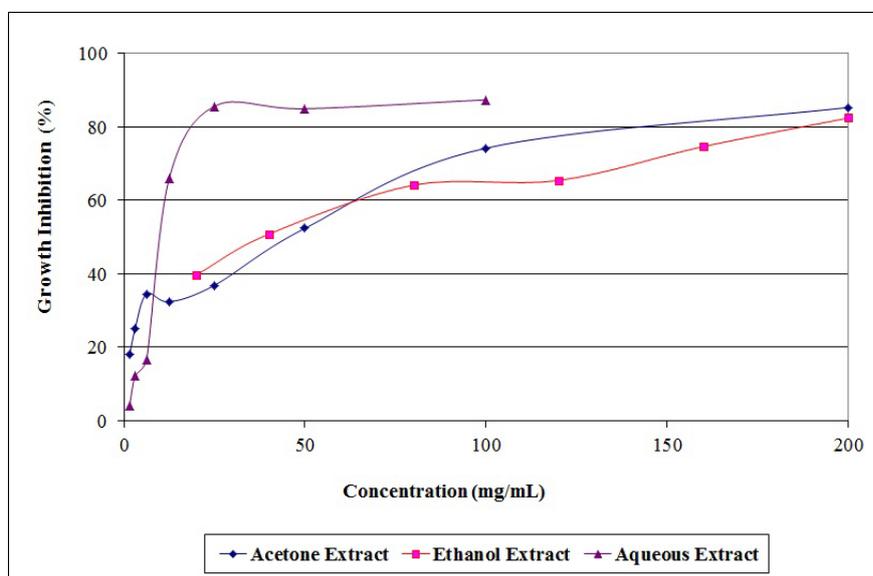

Fig. 1: Percentage of growth inhibition of *Plasmodium falciparum* strain D10 after incubation of *Peronema canescens* leaves extract for 72 h. *Tests were performed for 6-8 concentration levels and 3 replications for each sample

The highest concentrations of the acetone, ethanol and aqueous extracts inhibited the growth of the FCR3 strain by 90.51±2.32%, 92.76±0.43% and 69.60±1.21%, respectively, after a 72 h incubation, as shown in fig. 2. The mean IC$_{50}$ values for the acetone, ethanol and aqueous extracts were 51.14±8.65, 70.22±14.13 and 34.85±6.04 µg/ml, respectively. The ANOVA test (p = 0.05) for the

FCR3 revealed a significant difference between the three extracts (p = 0.00). *Post-hoc* test results showed significant differences between the ethanol and aqueous extracts (p = 0.01), but no significant differences between the acetone and ethanol extracts (p = 0.15) or between the acetone and aqueous extracts (p = 0.24) in terms of their ability to inhibit the growth of the FCR3 strain.
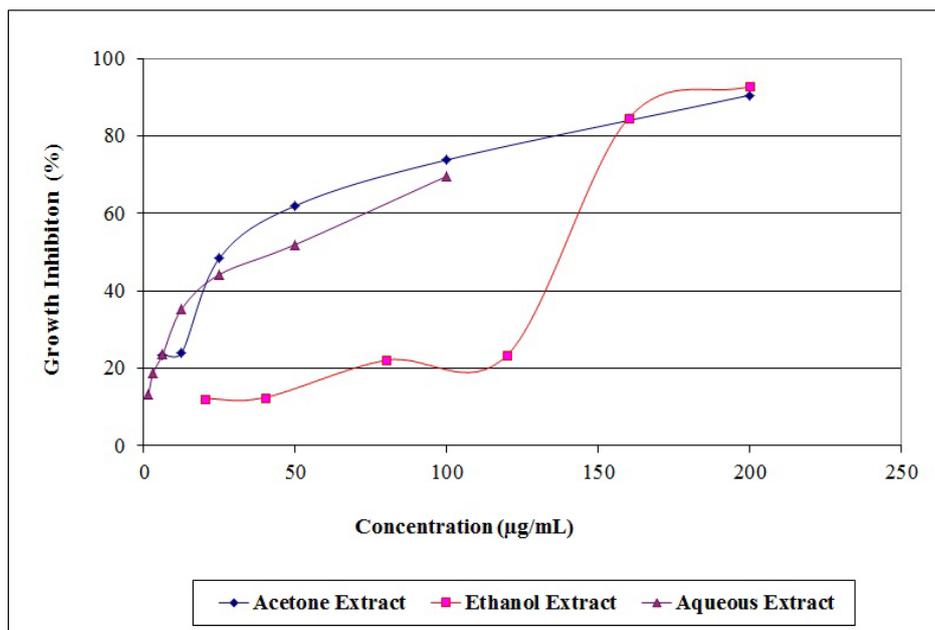
Fig. 2: Percentage of growth inhibition of *Plasmodium falciparum* strain FCR3 after incubation of *Peronema canescens* leaves extract for 72 h. *Tests were performed for 6-8 concentration levels and 3 replications for each sample

The IC_{50} values for *Plasmodium falciparum* strains D10 and FCR3 indicated that the *Peronema canescens* leaf extracts were more effective against *Plasmodium falciparum* strain D10 than against *Plasmodium falciparum* strain FCR3 (table 1).

Table 1: Antiplasmodial activity (IC_{50} in µg/ml), cytotoxicity activity (IC_{50} in µg/ml) and Selectivity Index of *Peronema canescens* leaves extract against *Plasmodium falciparum* D10 and FCR3 strains and vero cell at 72 h Incubation

Extract	Vero Cell*)	*Plasmodium falciparum* D10 strain*)	Selectivity index	*Plasmodium falciparum* FCR3 strain*)	Selectivity index
Acetone	23.37±5.63	26.33±1.65	0.89	51.14±8.65	0.46
Ethanol	629.46±24.85	37.96±8.17	16.46	70.22±14.13	8.90
Aqueous	634.00±144.82	12.26±1.05	51.70	34.85±6.04	18.00

*mean±SD, **Tests were performed for 6-8 concentration levels and 3 replications for each sample

Cytotoxicity activity

The acetone extract at the highest concentration (400 µg/ml) was highly cytotoxic, causing the death of 98.26±0.67% of the Vero cells. Concentrations of 200 µg/ml caused the death of 99.10±1.56% of the cells. By contrast, the ethanol and aqueous extracts, at the same highest concentration (400 µg/ml) resulted in the death of only 11.34±6.38% and 8.69±6.32% of the Vero cells, respectively. The IC_{50} values for the acetone, ethanol and aqueous extracts were 23.37±5.63, 629.46±24.85 and 634.00±144.82 µg/ml, respectively (table 1). Statistical analysis using ANOVA (p = 0.05) revealed a significant difference between the three extracts (p = 0.00).

Selectivity index

The level of drug toxicity can be compared to its activity based on the selectivity index or the cytotoxicity index. In this study, the selectivity index was calculated by comparing the IC_{50} value from the cytotoxicity test and the IC_{50} of the test activity at 72 h of incubation. The selectivity index values are shown in table 1.

DISCUSSION

The IC_{50} values for the different *Peronema canescens* leaf extracts can be grouped according to the criteria of the observed antiplasmodial activity for herbal extracts [20–22]. Based on these criteria, all *Peronema canescens* leaf extracts had good antiplasmodial activity against *Plasmodium falciparum* strain D10. However, the activity against *Plasmodium falciparum* strain FCR3 was only evident with the aqueous extract, as the acetone and ethanol extracts showed poor antiplasmodial activity. Therefore,

aqueous leaf extracts are likely to show better antiplasmodial activity overall when compared to acetone and ethanol extracts.

Previous research on *Peronema canescens* has indicated that acetone extracts have antiplasmodial activity against *Plasmodium falciparum* strain K1, and that this activity may be due to the presence of 7-clerodane diterpenoid peronemins found in the acetone extracts. Six of these compounds have been tested and two, peronemin C1 and peronemin B1, had antiplasmodial activity. The IC_{50} value of peronemin C1 was 13.1 µM (5.03 µg/ml), and peronemin B1 at a dose of 118 µM (43 µg/ml) could inhibit plasmodial growth by as much as 83% [23]. The difference between these results and those of the present study may reflect differences in the tested strains of *Plasmodium falciparum*. For example, the *P falciparum* D10 strain was less sensitive to the acetone and ethanol extracts, but these extracts still had some antiplasmodial activity.

Aqueous extracts have also been reported previously to have antiplasmodial activity. A study on *Vernonia cinerea* identified the antiplasmodial compound in an aqueous extract as a sesquiterpene lactone [24]. Other research on *Peronema canescens* leaves indicated that an aqueous extract had *in vitro* activity against *Babesia gibsoni,* with an IC_{50} value of 43.8±3.5 µg/ml [14]. Other research has also indicated that aqueous extracts from various plants show antibacterial and antiplasmodial activity [8].

The antiplasmodial activity of a natural product has to be balanced by low cytotoxicity. In the present study, an $IC_{50}>30$ µg/ml was considered to indicate low toxicity and an IC_{50} of 20–30 µg/ml was considered to indicated moderate cytotoxicity [21]. Based on this

categorization, the acetone extract has moderate cytotoxicity, and the ethanol and aqueous extracts have low cytotoxicity. The *post-hoc* test indicated that the acetone extract is more toxic than the aqueous and ethanol extracts, while the aqueous and ethanol extracts have almost the same cytotoxicity. Research on the cytotoxicity of sungkai leaf extracts has not been widely reported, although one *in vivo* toxicity test of a *Peronema canescens* extract found that no mice that died after the test [14]. Another study on *Peronema canescens* leaves showed that a methanol extract and an N-hexane fraction of *Peronema canescens* leaves gave LC_{50} values of 387.257 and 107.399 μg/ml, respectively, against shrimp larvae [25].

Any drug used to treat parasite infections must be selective and only inhibit the biological processes essential to parasite development without affecting the host. The selectivity index, which is the ratio of the IC_{50} for cytotoxicity over the IC_{50} for antiplasmodial activity, is a useful parameter for determining the selectivity of the three extracts. A selectivity index ratio of less than 10 indicates a non-selective drug [21]. The selectivity index for the acetone extract indicated that it is not selective, whereas the ethanol and aqueous extracts are selective. The aqueous extract of *Peronema canescens* leaves would appear to be a better choice for development as an antimalarial drug because it had the best antiplasmodial activity and the best selectivity. These results of this study differ from those previously published, which suggested that peronemins isolated from acetone extracts have antiplasmodial activity [23]. In the present study, the acetone extract had good antiplasmodial activity but was highly toxic. An antiplasmodial should have good selectivity for the parasitic cells without harming the host cells. Another study has also reported that an ethanol extract has antiplasmodial activity against mice infected with *Plasmodium berghei* [13]. In the present study, the ethanol extract had the lowest antiplasmodial activity when compared with the other two extracts, although its toxicity was also low. The results of the present study are in line with the habits of people who currently use decoctions of *Peronema canescens* leaves to treat malaria and fever.

CONCLUSION

The aqueous extract of *Peronema canescens* leaves had the highest *in vitro* antiplasmodial activity and the best selectivity index.

AUTHORS CONTRIBUTIONS

1. Conceptualization and study design–Jhons Fatriyadi Suwandi, Mahardika Agus Wijayanti, Mustofa.
2. Data collection, sample analysis and performing the experiment design–Jhons Fatriyadi Suwandi, Mahardika Agus Wijayanti, Mustofa
3. Preparation of manuscript design–Jhons Fatriyadi Suwandi
4. Statistical Analysis design–Jhons Fatriyadi Suwandi.
5. Proofreading the manuscript design–Jhons Fatriyadi Suwandi
6. All authors read and approved the final manuscript.

CONFLICTS OF INTERESTS

All authors have not conflicted of interest

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